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Original Research

Identification of a novel missense eya4 mutation causing autosomal dominant non-syndromic hearing loss in a Chinese family

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Abstract: The aim of this study was to identify the novel missense eya4 mutation which cause autosomal dominant non syndromic hearing loss In a Chinese family. Hearing loss is the most common sensory deficit in humans, but the middle-frequency sensorineural hearing loss (MFSNHL) is rare among hereditary non-syndromic hearing loss, and EYA4 is one of the genes reported to be associated with MFSNHL. A genetic analysis of a Chinese family with autosomal dominant non-syndromic progressive hearing impairment was conducted and assessed. Targeted exome sequencing, conducted using DNA samples of an affected member in this family, revealed a novel heterozygous missense mutation c.1855T>G in exon 20 of EYA4, causing amino-acid (aa) substitution Gly for Trp at a conserved position aa-619. The p.W619G mutation related to hearing loss in this Chinese family was validated by Sanger sequencing. Bioinformatic analysis confirmed the pathogenic effects of this mutation. We identified the novel missense mutation c.1855T>G (p.W619G) in EYA4 causing autosomal dominant non-syndromic hearing impairment in the selected Chinese family.

Key words: EYA4; Hearing loss; Targeted exome sequencing; Mutation.

Introduction

Hearing loss is the most common sensory disorder in human beings. It can be caused by genetic and environmental factors, such as medical factor, environment exposure, injury and medicine. Among the hereditary hearing loss, 70% is non-syndromic, which is human monogenic inherited disease. Among these patients, the middle-frequency (500-2000Hz) sensorineural hearing loss (MFSNHL) is rare. And only three genes are associated with MFSNHL, including TECTA (DFNA8/12) (1, 2), EYA4 (DFNA10) (3) and COL11A2 (DFNA13) (4, 5). Among these genes, “Eyes absent 4” (*EYA4*), identified by Wayne S et al (6), a member of the vertebrate EYA family of transcriptional activators, is the causative gene for DFNA10. Encoding a 639 amino acid protein, EYA4 is composed of a highly conserved 271 amino acid carboxy terminus called the eya-homologous region (eyaHR) and a more divergent proline-serine-threonine (PST)-rich domain at the amino terminus called the eya-variable region (eyaVR) (7). Mutations in the EYA genes have been associated with several congenital diseases, including congenital cataracts (8), a multi-organ disease called branchio-oto-renal syndrome (9) and late-onset deafness (6, 10-12).

Thus far, 16 mutations have been described with mutations in *EYA4* that cause hearing impairment (Table 1). Mutations in the EYA4 gene cause inherited DFNA10 autosomal dominant hearing loss (6), which shares a similar phenotype: late-onset, progressive, sensorineural hearing loss (SNHL), age of onset varying from 6 to 50 years old; at onset, hearing losses were mainly situated at the midfrequencies; with increasing age, all frequencies became affected; the hearing loss was initially mild, with a spontaneous evolution to a moderate or severe hearing impairment (13).

Here, We report the application of targeted exome capture to identify a novel missense mutation c.1855T>G (p.W619G) in *EYA4* in a family, shedding new light on the pathogenic mechanism of *EYA4* mutations.

Materials and Methods

Family and clinical evaluation

A Chinese family with late onset progressive sensorineural hearing loss was ascertained in our hospital. The pedigree of this family, spanning 4 generations and including 21 members, is consistent with an autosomal dominant inheritance pattern. 12 family members, including 3 affected and 9 unaffected individuals, participated in this study. The study was approved by the ethics committee of Chinese Beijing Luhe Hospital Capital Medical University and appropriate informed consent was obtained from all participants accordingly. Medical history was obtained by using a questionnaire regarding the following aspects of this condition: subjective degree of hearing loss, age at onset, evolution, symmetry of the hearing impairment, use of hearing aids, the presence of tinnitus, pressure in the ears or vertigo, medication, noise exposure, pathological changes in the ear and other relevant clinical manifestations. Otoscopy, physical examination and pure tone audiometry (at frequencies from 250 to 8000Hz) were performed to identify the phenotype. Acoustic immittance testing was applied

Table 1. Summary of clinical data for affected individuals of this family.

		Age (years)		Pure-tone average$_a$ (dBHL)			
Subjects	Gender	At testing	At onset	Left	Right	Audiogram shape	Degree of hearing loss
II-2	Female	62	51	71.25	76	Flat-sloping	Severe
II-4	Male	68	53	98.75	93	Flat	Severe
III-9	Male	44	41	38	36	Valley	Mild

Pure-tone average was calculated by hearing threshold levels in speech frequencies 0.5, 1, 2 and 4 kHz.

to evaluate middle-ear pressures, ear canal volumes and tympanic membrane mobility.

Next generation sequencing and analysis

The blood samples were collected from these patients. RNA-free high-molecular-weight DNA were prepared by using the Blood DNA Extraction Kit. The quality and concentration of genomic DNA sample was assessed by agarose gel electrophoresis and NanoDrop spectrophotometer. For genes from nucleus genome, 1500ng genomic DNA was fragmented into an average size of 300bp, then the fragmented genomic DNA was used for preparation of sequencing libraries 8bp barcoded sequencing adaptors were then ligated with DNA fragments before final hybridization with 6110 genes related to inherited diseases focused exome probes. The quality and quantity were measured by both AATI fragment analyzer and qPCR. Purified sequencing libraries were pooled together and massively parallel sequenced by Illumina HiSeq X ten platform to produce an average XGb per sample with average sequencing depth around 150×, respectively. These data were then aligned to the hg19 genome using NextGENe, an alignment method that is similar to BLAT methodology to align sequence reads to the reference. NextGene uses a preloaded index alignment algorithm that employs a suffix array that is represented by the Burrows-Wheeler Transform (BWT). All the mutations were compared with databases such as dbSNP, ExAC or 1000 genome projects and the frequency of occurrence were then calculated in different population group. Pathogenicity predication analysis was carried out on platforms of SIFT, Polyphen-2 and mutation taster.

Sanger sequencing

All the mutations found that can potentially cause the diseases were subject to sanger sequencing (ABI3130) for verification.

Results

Clinical feature of the family

The Chinese family showed an autosomal dominant inheritance pattern (Figure 1). According to information collected from questionnaires, the age at onset of hearing impairment varied from 40 to 60 years-old in this family. In all 3 affected subjects, the hearing impairment was symmetric while the severity varied according to the audiograms (Table 1). The hearing loss first affected the middle frequencies (<2000Hz). Audiologic evaluation of the family members demonstrated normal acoustic immittance testing and bone conduction values that equal the air conduction measurements, suggesting sensorineural hearing impairment. No patients had a history of the use of aminoglycosides and only one patient (III-9) had excessive exposure to noise for about 20 years. Computed tomography scan analysis of the proband of the family had been conducted to rule out inner-ear malformations. Comprehensive family medical history and clinical examination of these individuals showed no other clinical abnormalities, including cardiovascular disease.

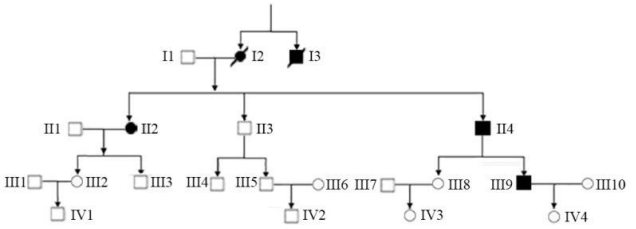

Figure 1. Pedigree of the family.

Targeted exome sequencing

99.724% of the targeted disease gene regions were sequenced, and 99.463% of the targeted bases were sequenced with 10X depth that allowed to accurately call a SNP. The variants were functionally annotated using an in-house pipeline as well as the reported results from public available databases (dbSNP 135, HapMapdatabase, 1000 genome variants database and a local control database) and categorized into different groups, including missense, non-sense, splice-site, insertion, deletion, synonymous and non-coding mutations. For all variants, the results were filtered with a quality value of single base sequencing ≥ 20. A total number of 25735 variants were identified in the sample. Among them, there were 3188 nonsynonymous variants, missense, non-sense and splicing variants (Table 2).

Sanger sequencing

We carried out Sanger sequencing and found a missense variant, c.1855T>G; p.W619G, in exon 20 of EYA4 [NM 004100.4, (MIM_603550)], which co-segregated with the disease (Table 3). This novel mutation

Table 2. Targeted exome sequencing.

sample	01170113100101
initial bases on target	17895567
base covered on target	17846176
coverage of target region	99.724%
Effective sequence on target (Mb)	28.94M
Fraction of effective bases on target	78.067%
Average sequencing depth on target	197.22
Fraction of target covered with at least 4X	99.644%
Fraction of target covered with at least 10X	99.463%
Fraction of target covered with at least 20X	98.912%

Table 3. Summary of the identified EYA4 mutations associated with the DFNA10 locus.

Family original/ Publication	Location	Nucleotide change	Amino acid change	Mutant type	Domain	Symtoms	Reference
America/2001	Exon 12	c.1468insAA	p.T343Kfs*62	Truncating mutation	eyaHR and eyaVR	SNHI	Wayne et al., 2001 (6)
Belgium/2001	Exon 20	c.C2200T	P.R587*	Truncating mutation	eyaHR	SNHI	Wayne et al., 2001 (6)
Hungary/2002	Exon 13	c.1558insTTTG	p.W374Cfs*6	frameshift mutation	eyaHR	SNHI	Pfeffer et al., 2017 (19)
America/2007	Exon 12	c.1490insAA	p.R352Pfs*53	frameshift mutation	eyaHR	SNHI	Makishima et al., 2007 (11)
Australia/2007	Intron 14	c.T1282-12A	P.Q427fs	frameshift mutation	eyaHR	SNHI	Hildebrand et al., 2007 (10)
Korea/2012	Exon 11	c.C863A	p.S288X	nonsense mutation	eyaHR	SNHI	Baek et al., 2012 (12)
Korean	Exon 12	c.978C>G	p.F326L	nonsense mutation	eya VR	SNHI	Choi et al., 2013 (25)
China/2014	Exon 15	c.T1301A	p.I411K	missense mutation	eyaHR	SNHI	Tan et al., 2014 (26)
Sweden/2015	Exon 8	c.579_580insTACC	p.D194Tyrfs*52	frameshift mutation	eyaHR	SNHI	Frykholm et al.,2015 (27)
Korea/2016	unknown	c.1194delT	p.Met401TrpfsX3	truncation mutation	eyaHR	SNHI	Choi et al.,2016 (28)
China/2015	Exon 8	c.G511C	p.G171R	missense mutation	eyaVR	SNHI	Liu et al., 2015 (29)
China/2015	Exon 18	c.C1643G	p.T548R	missense mutation	eyaHR	SNHI	Sun et al.,2015 (30)
Korea/2015	Exon 13	c.C1177T	p.Q393X	nonsense mutation	eyaHR	SNHI	Kim et al., 2015 (31)
Dutch/2016	Exon 8	c.464del	p.Pro155fsX	truncating mutation	eyaHR	SNHI	van Beelen et al., 2016 (32)
China/2015	Exon 8	c.544_545insA	p.F221X	frameshift mutation	eyaHR	SNHI	Huang et al., 2015 (33)
America/2005	Exons 9 and 10	4846-bp deletion	p.Asp194Glyfs*30	frameshift mutation	SNHI and dilated cardiomyopathy		Schönberger et al., 2005 (20)

Table 4. Software predicting results.

Gene	EYA4
Gene OMIM no	603550
Disease OMIM no	601316
Chromosome coordinates (GRCh37/hg19)	Chr6:133849878
Nucleotide	c.1855T>G
NM	NM 004100.4
Area	CDS19
homozygous/ heterozygous	heterozygous
Amino Acid	p.W619G
MAF	N/A
Pathogenicity analysis	suspected pathogenic mutations
Prediction information	SIFT: Damaging Polyphen2: Probably Damaging Mutation Taster: Disease causing
Disease phenotype	Autosomal dominant deafness type 10
Genetic way	AD

was identified in 6 patients (II-2, II-4, III-2, III-8, III-9, IV-1) but 3 patients have no symptoms of deafness yet, and their onset of hearing loss is anticipated during follow-up.

In silico analysis

To understand the potential effect of the W619G missense mutation on EYA4 function, we further performed in silico analyses. This mutation was predicted to be "Damaging", "Probably Damaging" and "Disease-causing" by SIFT, Polyphen2 and Mutation Taster shown in Table4, respectively. This finding indicated that this novel mutation might be the cause of the observed hearing loss in this Chinese family.

Discussion

In 2013, the World Health Organization estimated

that 360 million people worldwide live with disabling hearing loss and that as the population ages, the global burden of disease attributable to deafness increases (14-16). Traditional methods of screening new disease-causing genes are expensive and time consuming; recently, sequencing technology has remarkably progressed. In this study, targeted exome sequencing was used to find the disease-causing gene of a Chinese family with hearing loss, and we identified the EYA4 exon 20 missense mutation in the affected cases.

EYA4 is an EYA family member. The EYA proteins are components of a conserved regulatory network involved in cell-fate determination in organisms ranging from insects to humans (17). In higher animals, this network is often referred to as the Pax-Six-Eya-Dach network (PSEDN) to better reflect the vertebrate genes/proteins involved. PSEDN is both a purely transcriptional and a signal transductional network. The eyaHR and SIX family transcription factors interact to form transcriptional complexes that regulate the expression of target genes required for the development and maturation of the organ of Corti (6). This encoded protein is also a putative oncogene that mediates DNA repair, apoptosis, and innate immunity following DNA damage, cellular damage, and viral attack (18).

Mutations in this gene are associated with postlingual, progressive, autosomal dominant hearing loss at the deafness autosomal dominant non-syndromic sensorineural 10 locus. So far, there have been 16 EYA4 mutations reported (Table 3). In the present family, patients have a similar phenotype as described before: late-onset, progressive, sensorineural hearing loss (SNHL); at onset, hearing losses were mainly situated at the mid-frequencies; the hearing loss was initially mild, with a spontaneous evolution to a moderate or severe hearing impairment. The novel c.1855T>G mutation creates a p.W619G substitution in the eyaHR, which is crucial for the function of the protein. The Tryptophan residue at 619 in EYA4 was highly conserved across species. According to Pfeffer CM et al (19), non-conservative substitutions at fixed or conservative sites and conservative substitutions at fixed sites are likely to affect function in humans. In this family, the difference was that the onset age was older (>40 years old) than studies ever reported. One patient (III9) had symptoms earlier (at 41 years old) than others may be because of the excess noise exposure. The reason of their later onset may be that different mutations could cause different changes of function of the protein. There were three phenotypically normal individuals (III-9, III-15 and IV-18), aged <40 in this family who tested positive for the *EYA4* mutation. Their onset of hearing loss is anticipated during follow-up, which may support the finding.

Many pathogenic mechanisms have been presumed to be involved in NSHL caused by EYA4 mutations, including loss of gene function and haploinsufficiency through reduced gene dosage, expression or protein activity. EYA proteins interact with members of SIX and DACH protein families in a conserved network that regulates the early embryonic development and post-developmentally continued function of the mature organ of Corti (6). The haploinsufficiency of EYA4 may lead to inadequate cochlear transcriptional regulation and function maintenance (10, 12), causing SNHL, even if the mutant proteins are present in the cells and partially functional (20). In addition, members of the EYA gene family were suggested to induce apoptosis by triggering the caspase-dependent and -independent pathways (21). Abnormal ear functions of cochlea and vestibule, have been shown in caspase-3-deficient mice (22). Thus, the apoptotic deficiency due to EYA4 mutation may also be involved in human DFNA10 hearing loss (23). Wang et al (24) indicated that Eya4 regulates Na+/K+-ATPase, which is crucial for the development of mechanosensory cells of the inner ear and the maintenance of cardiac function in zebrafish, which potentially provides a mechanism by which human EYA4 mutations cause hearing loss and heart disease.

Defects in this gene are also reported to be associated with dilated cardiomyopathy. There is a hypothesis that the deafness phenotype (syndromic or non-syndromic) is correlated with the *EYA4* mutation position: truncations affecting only the EYA domain of the protein cause SNHI alone, whereas truncations affecting the more N-terminal variable region of the protein lead to both SNHI and DCM (11, 20). Studies about zebrafish confirmed that *EYA4* has a role in cardiac muscle by assessing the effects of attenuated *EYA4* expression in zebrafish and *EYA4* regulation of NA+/ K+-ATPase is crucial for the development of mechanosensory cells and cardiac function in zebrafish (20). In the present family, in the absence of a family history of heart failure and premature death, a severe form of DCM is very unlikely, and there is no evidence for any cardiac problems, inferring that mutations only affects the EYA domain. To determine the mechanism of deafness caused by gene mutation, and whether it would lead to DCM, we will next carry out animal experiments and more research on the cell level.

We found a new mutation that led to DFNA10 deafness, but the study had a few shortcomings, such as failure to perform audiological tests (such as otoscopy, pure tone audiometry and acoustic immittance testing) and other related tests (such as brainstem auditory evoked potential) because of the rejection of some family members, which is a great regret. In addition, these cases of missense eya4 mutation causing non-syndromic hearing loss is relatively rare, the data of this study are only from one Chinese family, and the small samples may lead to some biases. In the next step, more similar cases should be collected to strengthen the correlation, further the animal studies and some research on cell level should be conducted in the future.

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None.

Conflict of interest
The authors declare that no conflict of interest is associated with this work.

Author contributions
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Rui-hua Zhang; Shuying Xiao, Jing Qu, Qin Zhang, Ting Ao, Jun Zhang, Rui-hua Zhang collected and analysed the data; Shu-

ying Xiao wrote the text and all authors have read and approved the text prior to publication.

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